

Does cotton bollworm show cross-resistance to the *Bacillus thuringiensis* toxins Cry1Ac and Cry2Ab? A mini review

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Abstract: Since 1996, transgenic *Bacillus thuringiensis* (Bt) cotton has been commercially grown in numerous countries in an effort to stem the losses caused by key lepidopteran pests. However, the development of pest resistance to Bt toxins has jeopardized the continued utilization of Bt cotton. As a strategy designed to circumvent the development of resistance, Bt cotton varieties expressing two or more toxins targeting the same pest have been introduced. Nevertheless, from the perspective of long-term planting of Bt cotton, the potential risk of cross-resistance to these Bt toxins is a threat that cannot be ignored. In this paper, we review current research (including that based on the analysis of protein binding sites and resistance genes) on the resistance of cotton bollworm (*Helicoverpa armigera*) to the Bt toxins Cry1Ac and Cry2Ab and the interrelationship between these toxins. On the basis of existing evidence, we assume that the actions of Cry1Ac and Cry2Ab against cotton bollworm are not completely independent, and then propose the "resistance-associated gene mutation potential hypothesis". Although the mechanisms underlying the resistance of pests to Bt toxins are yet to be comprehensively elucidated, this hypothesis could undoubtedly have important implications for adopting "pyramid" strategy in the future. Further research is recommended to devise strategies to retard the development of *H. armigera* resistance to Bt cotton, either using different Bt toxins or their various combinations.

Keywords: cross-resistance; genetically modified cotton; cotton bollworm; *Bacillus thuringiensis*; Cry1Ac; Cry2Ab

1 Introduction

The development of genetically modified cotton was stimulated to a large extent by the burgeoning resistance of lepidopteran pests to conventional chemical pesticides. The long-term extensive use of chemical pesticides to control cotton bollworm (*Helicoverpa armigera*) provided intense selection pressure that resulted in the rapid evolution of resistance to these pesticides, and as a consequence led to an escalation in pesticide application rates (Luttrell et al., 1994; Wu et al., 2005; Tay et al., 2015; Fleming et al., 2018). This overuse resulted in higher control costs and severe environmental pollution, whereas resistance to the pesticides posed a considerable threat to

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cotton production (Wu and Guo, 1997, 2005). In an effort to retard or even prevent the development of cotton bollworm resistance to pesticides, cotton was genetically modified to produce insecticidal proteins that would specifically target lepidopteran pests (Sanahuja et al., 2011).

Bacillus thuringiensis (Bt) cotton has to date been commercially cultivated in numerous countries worldwide, including Brazil, Argentina, India, China, Paraguay, Pakistan, South Africa, Australia, Burkina Faso and the United States of America (ISAAA, 2018). Given its low cost and high yields, the area planted with Bt cotton, like that of other genetically modified crops, has increased rapidly (Shivendra and Deepthi, 2016), and by 2017, the transgenic crops were grown commercially on no less than 18.90×10^7 hm² in 24 countries, for example, 2.41×10^7 , 9.41×10^7 , 5.97×10^7 and 1.02×10^7 hm² of genetically modified cotton, soybean, maize and canola, respectively (ISAAA, 2018).

With the rapid and extensive commercialization of Bt cotton over the years, comprehensive resistance management methods and programs, including "pyramid" strategy, gene promoter and expression analyses, and field cultivation measures (Wang and Dai, 2009), have attracted the considerable attention of governments and scholars (Qiao et al., 2017; Wilson et al., 2018). In this regard, however, "pyramid", one of the most important management strategies (Sushmita et al., 2016), has emerged as a controversial issue with respect to the potential evolution of cross-resistance to different Bt genes by pests (Luo et al., 2007; Zhao, 2012; Kain et al., 2015; Tay et al., 2015; Wang et al., 2016; Shabbir et al., 2018). The development of resistance by pests can be achieved via a number of different mechanisms, including modification of Cry toxin receptors, the elimination of toxins from the cytosol, and the development of regulatory mechanisms that disrupt the production of toxin receptors (Melo et al., 2014). Furthermore, due to the complex nature of biological mechanisms and the heterogeneity of field environments, it is exceedingly difficult to completely clarify the mechanisms underlying the cross-resistance of pests to Bt toxins (Liu et al., 2018).

With a view toward gaining a better understanding of the mechanisms of cross-resistance evolved by pests, we herein put forward an explanatory hypothesis based on the research to date, namely, the "resistance-associated gene mutation potential hypothesis". It is our hope that this hypothesis and our reasoning could contribute to retarding the development resistance to Bt toxins by pests and promote a more rational and sustainable utilization of genetically modified crops.

2 Threat posed by pest resistance to the sustainable use of Bt cotton

The widespread planting of transgenic cotton is mainly driven by higher yields and lower costs, and thus gaining more substantial economic benefits (Pray et al., 2001; Qaim, 2009; Subramanian and Qaim, 2010). However, the value of Bt cotton could be diminished by insect resistance to Bt toxins. Many Cry1Ac- and Cry2Ab-resistant strains of *H. armigera* have been detected in different cotton planting areas (Akhurst et al., 2003; Liang et al., 2008; Nair et al., 2010; Wei et al., 2015; Cui et al., 2018). For instance, research results showed that cotton bollworm has developed field resistance to Cry1Ac in Pakistan (Alvi et al., 2012). Therefore, this transgenic technology is faced with the same problems that have plagued the use of chemical pesticides (Downes et al., 2017). Lack of refuges, the irrational use of unknown varieties and incomplete routine monitoring programs are among the most probable explanations for the evolution of field resistance (Tabashnik et al., 2009a) and pose a considerable threat to the sustainable utilization of Bt cotton (Allah et al., 2009).

3 Cotton bollworm resistance management strategies

In an effort to retard the increase in resistance to Bt toxins, a range of integrated resistance management strategies have been initiated (Gould et al., 1997; Gould, 2000; Xu et al., 2006), which can be divided into the following four categories (Wang et al., 2009): (1) gene strategies,

the introduction of single gene or multiple genes encoding toxin proteins that bind to different receptors (Niu et al., 2013; Carrière et al., 2018; Reisig et al., 2018); (2) gene promoters, tissue-specific expression of Bt genes under specific environmental conditions to control pests (McGaughey and Whalon, 1992; Williams et al., 1992); (3) gene expression, the maintenance of a high expression level of toxins in crops to kill all pests with hybrid resistance genes (Denholm and Rowland, 1992; Tabashnik, 1994); and (4) field tactics, maintaining a sensitive population of pest through uniform individual genes, multiple genes, gene rotation, proportional cultivation, non-Bt-producing crop refuges or "natural refuges" of traditional host plants with small-scale mixed planting patterns (Downes et al., 2007; Wu et al., 2008; Gujar et al., 2010).

Since 2004, with a view toward to impeding the evolution of resistance, Cry2Ab has been widely utilized for the control of cotton bollworm populations, typically in combination with Cry1Ac (Li et al., 2006; Hernadez et al., 2013). As a necessary proactive resistance management method, Cry1Ac and Cry2Ab sensitive baselines of field pest populations have been monitored in the main Bt cotton cultivated countries, including China, India, Brazil, Australia and the United States of America (Wu et al., 1999; Sivasupramaniam et al., 2007; Brvault et al., 2009; Kranthi et al., 2009; Kaur et al., 2011; Albernaz et al., 2013).

4 Cross-resistance to Cry1Ac and Cry2Ab

Some studies have showed that cotton bollworm does not show cross-resistance to Cry1Ac and Cry2Ab (Luo et al., 2007). Cry2Ab resistance in cotton bollworm could be attributed to three independent resistance-related insertions and deletions in an ATP-binding cassette transporter gene (Tay et al., 2015). Cadherin proteins may be closely associated with the Cry1Ac resistance of cotton bollworm (Xu, 2005; Yang, 2010). No cross-resistance to Cry1Ac is observed in isolated Cry2Ab-resistant mutant lines due to differences in the mechanisms of actions of Cry1Ac and Cry2Ab (Tay et al., 2015), and binding site analyses in two *Helicoverpa* species have accordingly indicated that colonies resistant to Cry2Ab show no cross-resistance to Cry1Ac. Furthermore, resistant pests with a significantly decreased binding between Cry2Ab toxin and midgut receptors have been found to show no comparable inhibition for Cry1Ac binding (Caccia et al., 2010). Thus, it is postulated that binding site mutation may be responsible for the occurrence of Cry2 protein resistance in *Helicoverpa* pest species. Conversely, bioassay and binding analyses in cotton bollworm have indicated that lines resistant to Cry1Ac are susceptible to Cry2Ab, and that there are differences in the binding sites of Cry1Ac and Cry2Ab (Luo et al., 2007). Consistently, other research results have suggested that Cry1Ac resistance may not induce a high cross-resistance to Cry2Ab (Tabashnik et al., 2002), and bioassay, *in vitro* labeling and surface plasmon resonance analyses have similarly revealed that there is no brush border membrane vesicle (BBMV) combining competition, and that Cry1Ac-resistant cotton bollworm shows no cross-resistance to Cry2Ab (Luo, 2007). By using protein analysis technology, Yin (2012) concluded that Cry2Ab and Cry1Ac have different binding sites on the BBMVs of cotton bollworm based on the excluding micro-effect competition. According to binding site analyses in *Ostrinia nubilalis* and *Spodoptera frugiperda*, cross-resistance between proteins (Cry1ab/ac, Cry1A.105 and Cry1Fa) and Cry2A proteins is very unlikely (Hernandez-Rodriguez et al., 2013). In contrast to the aforementioned studies, however, other researchers proposed that many lepidopteran pests, including the cotton bollworm, have developed cross-resistance to Cry1Ac and Cry2Ab (Yu, 2004; Luo, 2007; Wei et al., 2012, 2015). For example, resistance evaluation in *Helicoverpa zea*, one of the most important target pests of pyramided Bt cotton, has revealed a low cross-resistance between Cry1Ac and Cry2Ab (Welch et al., 2015).

Laboratory diet experiments using the cotton bollworm have indicated that under the selection of Cry1Ac, pests exhibit a low cross-resistance to Cry2Ab (Liu et al., 2018). The findings of previous studies have also showed that dual-toxin Bt cotton expressing Cry1Ac and Cry2Ab would be more effective in cases that no Bt spray or only Cry1Ac Bt spray is used, compared with the use of Cry2Ab Bt spray (Wei et al., 2015). Nevertheless, two cotton bollworm Cry1Ac-resistant strains collected from fields in northern China were found to have a

simultaneous cross-resistance to Cry2Ab (Jin et al., 2013). Other insect pests, including *H. zea*, have also been found to show a developed cross-resistance to Cry1Ac and Cry2Ab, thereby markedly reducing the efficacy of the pyramid strategy for pest control (Brevault et al., 2013). For *Pectinophora gossypiella*, a laboratory Cry2Ab-resistant strain, has been shown to have a high cross-resistance to Cry1Ac; although laboratory Cry1Ac- and Cry2Ab-resistant strains were found to be able to survive on cotton producing Cry1Ac, they were incapable of surviving on pyramided Bt cotton (Tabashnik et al., 2009b). Zhao (2012) previously reported that Cry2Ab-resistant cotton bollworm strains had a cross-resistance to Cry1Ac.

The asymmetrical cross-resistance might help the sustainable use of Bt cotton containing Cry1Ac and Cry2Ab. And the synergistic actions of Cry1Ac and Cry2Ab against cotton bollworm suggest that the efficacy of pyramided Bt cotton against bollworm could be prolonged (Wei et al., 2015). However, these above findings accordingly imply that such cross-resistance might accelerate the development of pest resistance to Bt biological pesticides and pyramided Bt crops (Welch et al., 2015). The resistance could conceivably be attributable to the occurrence of resistance to pyramided Bt cotton caused by the screening of one of these toxins that were applied in the field. Given the selective pressures of Bt biocides used in the field, insect populations could evolve resistance to pyramided Bt crops (Kain et al., 2015).

5 The "resistance-associated gene mutation potential hypothesis"

Although numerous studies have investigated the mechanisms underlying the resistance of *H. armigera* to Bt toxins from ecological, physiological and molecular biological perspectives (Wei et al., 2016; Zhao et al., 2016; Liu et al., 2017; Xiao et al., 2017), these mechanisms are presumed to be complex and diverse, and accordingly are yet to be completely clarified (Peterson et al., 2017). Indeed, there is still a general lack of consensus regarding the occurrence of cross-resistance, particularly in the case of cotton bollworm with respect to the two widely used Bt toxins, i.e., Cry1Ac and Cry2Ab (Luo et al., 2007; Zhao, 2012; Welch et al., 2015). A possible explanation for the disparity among research findings could be that resistance-associated genes are not confined to a single specific site or a few sites, but involve a diverse range of sites with different genetic mutation potentials. Given this scenario, we propose a new hypothesis, namely, the "resistance-associated gene mutation potential hypothesis". Essentially, this hypothesis states that for a specific pest species, including the cotton bollworm, genetic mutation associated with the resistance to an environmental stress might occur at many sites based on the differences in resistance mechanisms and biochemical pathways, and that binding sites involved could conceivably have different mutation potentials. When certain resistance-associated sites do not mutate or when mutations that do occur are ineffective in conferring resistance to stress, other resistance-associated sites could mutate with a higher fitness cost, such as higher mortality and longer evolutionary processes. These processes would continue until the population either develops resistance or dwindles to extinction.

6 Consistency between hypothesis and current research results

On the basis of the aforementioned hypothesis, we here attempt to provide a rational explanation for the seemingly contradictory research findings.

6.1 Resistance-associated binding sites and mutational diversity

The activity of Bt toxins is generally mediated via a series of physiological and biochemical reactions, including dissolution in the alkaline conditions within the intestines, protease activation, the binding to receptors and glycosylphosphatidylinositol (GPI) anchor proteins, membrane perforation or signal transduction, and cell apoptosis (Bravo et al., 2008). Accordingly, resistance might evolve in response to changes in any one or a combination of these steps or the associated functional biochemical reactions, including toxin dissolution, enzyme activation, receptor binding and signal transmission. Considering this potential diversity of

resistance-evolving pathways, we propose that pests might adapt to a variety of complex environmental stressors, with the lowest ecological cost.

6.2 Asymmetrical cross-resistance of pests

Even when using the same experimental materials, test pests and methods, the results of studies might be completely different when the experiment is conducted forwardly and inversely. For example, a Cry1Ac- and Cry2Ab-resistant strain of *Pectinophora gossypiella* was found to survive on Cry1Ac cotton, but not on pyramided Bt cotton (Tabashnik et al., 2009). As a consequence of multi-generation feeding with Cry1Ac, cotton bollworm can develop a high resistance to Cry1Ac but retain a low resistance to Cry2Ab (Wei et al., 2015). However, multi-generation feeding with Cry2Ab resulted in a low resistance to Cry2Ab and a high resistance to Cry1Ac (Wei et al., 2015). Furthermore, in heterologous competition assays, Cry1Ac binding bands were found to be almost unaffected by the addition of 200 times unlabeled Cry2Ab, whereas all the Cry2Ab bands disappeared in the presence of 200 times Cry1Ac competition, thereby indicating significant differences in the binding proteins (Yin, 2012).

6.3 Genetic instability of cross-resistance

For cotton bollworm, the inheritance of resistance is known to be unstable. The resistance level of pests could decline under certain circumstances, whereas it could remain stable in other pest species. For instance, in the absence of Cry1Ac selection, the evolved rate of resistance has been observed to decline significantly over five generations (Li, 2009), which indicated that pest resistance to Cry1Ac and Cry2Ab can decrease within a non-Bt environment.

6.4 Effect of different binding characteristics

The binding sites or receptors for Bt toxins are known to have specific binding characteristics. Moreover, even in the case of the same site, different research methods can yield contrasting results. Accordingly, with respect to the cross-resistance of cotton bollworm to Cry1Ac and Cry2Ab, we could indeed draw seemingly contradictory conclusions, depending on the different research levels or perspectives. However, these conclusions could be more consistent if we take a holistic approach. For instance, certain results indicate that the occurrence of Cry2Ab resistance in cotton bollworm can be attributed to three genetic mutations in an ATP-binding cassette transporter gene, and that there is no cross-resistance to Cry1Ac in mutant strains as a consequence of differences in the action patterns of Cry1Ac and Cry2Ab (Tay et al., 2015). However, the results of other studies show that the binding of Cry2Ab to midgut proteins is disrupted in the presence of Cry1Ac toxin (Yin, 2012). These apparently contradictory results could be understood from a macro-perspective; specifically, the action of these two toxins is associated with independent resistance-related mutations, whereas they perhaps also have dependent competitive binding sites. In short, the conclusions drawn from these studies may each be valid in their own settings when targeting one or more binding sites or biochemical processes.

7 Conclusions

On the basis of the "resistance-associated gene mutation potential hypothesis" presented here, we recommend that pyramided Bt cotton strategies and their long-term efficacy should be reconsidered. Considering the substantial biological control value of these toxins, the primary goal of resistance management lies in the maintenance of their efficacy for as long as possible, considering the fact that the effectiveness of Bt cotton depends on the sensitivity of pests to these toxins. Given the various strands of evidence obtained from the research conducted to date, we can conclude that, with respect to cotton bollworm, the mechanisms of pest resistance to Cry1Ac and Cry2Ab are not completely independent. Accordingly, the potential risk of resistance to pyramided transgenic cotton might be considerably higher than initially anticipated.

However, although compared with single-toxin transgenic cotton, pyramided transgenic cotton has a superior control efficiency and a more durable "service" life, it is important to realize that the full potential of individual toxins should be developed when several toxins are simultaneously

used in the field. Therefore, resistance management measures could not be ignored and the greater value of all utilized Bt toxins should be realized. In this regard, the rotation of different single-toxin Bt crops and the rotation of these crops with non-toxin Bt should be considered as strategies to delay the evolution of resistance. Moreover, when introducing further toxins into genetically modified crops, we should pay more attention to integrated control measures for the rational utilization of Bt toxin resources.

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